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In-vitro virucidal and virustatic anti HIV-1 effects of extracts from *Persea americana* Mill. (avocado) leaves

M.D. Wigg[1,2], A.A. Al-Jabri[2,5], S.S. Costa[3,4], E. Race[2], B. Bodo[3] and J.S. Oxford[2]

[1]*Departamento de Virologia, Universidade Federal do Rio de Janeiro, Caixa Postal 68040, CEP 21941 590, Rio de Janeiro, Brazil.*

[2]*Academic Virology and Retroscreen Ltd, The London Hospital Medical College, University of London, UK.*

[3]*Nucleo de Pesquisa de Produtos Naturais, UFRJ, CCS, Bloco H, CEP 21941–590, Rio de Janeiro, Brazil.*

[4]*Laboratoire de Chimie, Museum National D'Histoire Naturelle, France.*

[5]*SQU, College of Medicine, Muscat,Sultanate of Oman.*

Summary

Aqueous (PA1) and methanolic extracts (PA2a–d; PA3) from the tropical tree *Persea americana* Mill. (Lauraceae), were evaluated for their cellular toxicity and anti-HIV-1 activity both in virustatic and virucidal assays. With the exception of PA3 and PA2d, all extracts showed anti-HIV-1 activity at concentrations which were not toxic for the H9 indicator cells. From the methanol insoluble extract (PA2) four different fractions (PA2a–d) were obtained using reverse-phase column chromatography, and two of the fractions (b and c) showed detectable virucidal effect. One fraction (PA2a) showed virustatic effects inhibiting HIV syncytium formation and viral p24 antigen formation at concentrations which were not toxic for the indicator cells. The results demonstrate for the first time that extracts from *P. americana* leaves have moderate anti-HIV-1 activity *in vitro*.

Key-words: antiviral activity; H9 cells; HIV; *in vitro;* plant extract.

Introduction

A number of specific antiviral drugs with virustatic activity have been approved for clinical use in patients with acquired immunodeficiency syndrome (AIDS), but the emergence of drug resistant viruses has imposed limitations on the efficacy of these compounds (Larder *et al.*, 1989; St. Clair *et al.*, 1991; Fitzgibbon *et al.*, 1992) leading to a renewed interest in virucidal agents (Oxford *et al.*, 1994). Virucidal drugs interact directly with viruses and cause virion destruction. In contrast, virustatic drugs normally act intracellularly and inhibit a vital virus-enzyme or virus-coded function. Virucidal compounds against HIV are being investigated widely for use as topically applied virus destructive drugs in the vagina and rectum to inactivate virus before adsorption to susceptible cells. The Plant Kingdom is an important source of molecules which have the ability to inhibit the replication of a range of viruses (Hudson, 1990; Che, 1991). Thus, Cheng and Yeung (1988) have demonstrated the inhibition of growth of HIV by crude extracts obtained from medicinal herbs, however, they did not study *Persea americana* species. An extensive diversity of plant species in Brazil with known medicinal value has been described by Rizzini and Mors (1976). As part of a project searching for novel virus inhibitors, and particularly those with virucidal activity which can be isolated, an extract obtained from *P. americana* leaves was studied. *Persea americana* Mill. (Family Lauraceae) is a tropical tree (avocado) which has been cultivated in Brazil. Avocado leaves have been used in traditional medicine as a diuretic and for removing kidney stones (Santos *et al.*, 1988). For the first time in-vitro anti-HIV-1 activity of the extract of leaves of this plant is described.

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Results

Figure 1 shows the absence of cytotoxic effects of the extracts and fractions of *P. americana* leaves on H9 cells. Thus, all drug samples showed equivalent to, or higher cell viability when compared to the control, with the exception of the PA3 extract which shows some toxicity only at 400 $\mu g\ mL^{-1}$.

The crude extract PA1 of the leaf showed a dose-dependent antiviral effect (Fig. 2). Thus, at non-toxic concentrations (100 $\mu g\ mL^{-1}$), the aqueous crude extract (PA1) of *P. americana* showed an $\approx 6\ \log_{10}\ TCID_{50}\ mL^{-1}$ reduction in virus infectivity end point titre. As a positive control for the test 5 μM AZT drug caused 1.75 $\log_{10}$ $TCID_{50}\ mL^{-1}$ reduction in the virus titre. Similarly, the methanol insoluble extract (PA2) had significant antiviral activity. However, the methanol soluble extract (PA3) had significantly less inhibitory effect on virus replication compared to the other extracts.

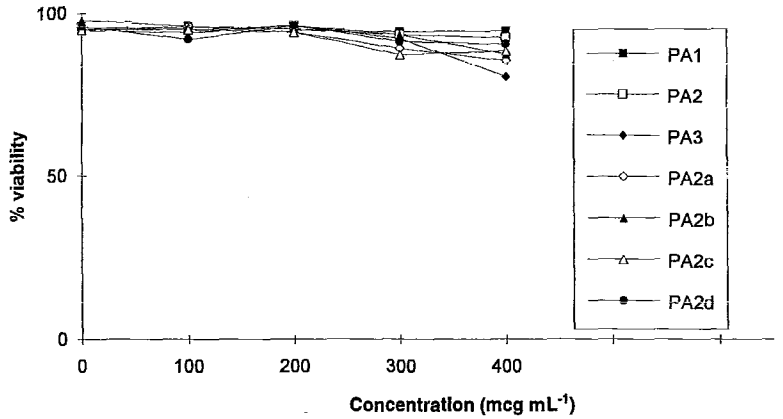

Fig. 1. Cytotoxic effects of *Persea americana* extracts and fractions.
H9 cells were incubated with either control medium or dilutions of *P. americana* extracts and fractions at 37 °C. All cytotoxicity tests were carried out in duplicate using 96-well plates. At day 8 after adding the extracts, the percentage of viable cells compared to the cell control in each well was calculated. The results are the mean of two experiments.

The results of a separate experiment to quantitate the antiviral effect of the extracts PA1, PA2 and the four fractions obtained from the methanol insoluble extract (PA2) using two different challenge concentrations of virus (10 and 100 $TCID_{50}$) are shown in Table 1. The ED_{50} varied among the extracts and was dependant on the infectivity titre of the challenge virus. Thus, when 10 $TCID_{50}$ of virus was used, fraction PA1 showed an ED_{50} of 18.7 μg mL^{-1}. However, 100 $TCID_{50}$ produced an ED_{50} of 33.7 μg mL^{-1}. In an assay which also quantitates effects of viral p24 antigen, 195 μg mL^{-1} of PA2a fraction caused 64% inhibition of antigen and 100% inhibition of HIV induced syncytia (Fig. 3).

Table 2 shows the results of the virucidal assay. Extracts PA1 and PA2 and fractions PA2b and PA2c exhibited a moderate virucidal effect, and no residual virus infectivity was detected after incubation of the extracts with virus for 1 h at 37 °C.

Discussion

Certain extracts and fractions of *P. americana* demonstrated antiviral effects at drug concentrations that did not exhibit any toxicity to H9 cells, even at concentrations as high as 400 μg mL^{-1} of the lyophilized extract. The inhibitory effect of the extracts was shown to be dose-dependent but moderate.

The specific antiviral effect was confirmed by demonstrating a reduction of the infectivity in virus titre in supernatants of drug treated but infected H9 cells.

The PA2a fraction showed particularly reproducible virustatic effects inhibiting both viral syncytium formation and viral p24 production. The same fraction has no virucidal activity. The PA2a fraction has been partially purified by chromatography and contains the flavonoid quercitrin as the major compound as well as another compound not yet identified. Quercitrin has previously been shown to have a moderate effect on the activity of HIV reverse transcriptase (Ono *et al.*, 1990). However, when pure quercitrin (Museum National D'Histoire Naturelle drugs collection) was analysed in the present study, no detectable antiviral activity was observed *in vitro* (data not presented). There are at least three possibilities which can be considered to explain the antiviral effect of the fraction PA2a, namely, (1) quercitrin present in the extract did not have any antiviral activity in H9 cells but might when tested in other cells; (2) quercitrin is acting in a synergistic way together with another compound that has not yet been identified; (3) the unidentified compound is the bio-active molecule. To confirm the last hypothesis it will be necessary to evaluate *P. americana* further in order to identify the putative second active compound in the PA2a fraction.

A wide variety of antiviral mechanisms are exhibited by compounds that can be found in plant extracts. These include

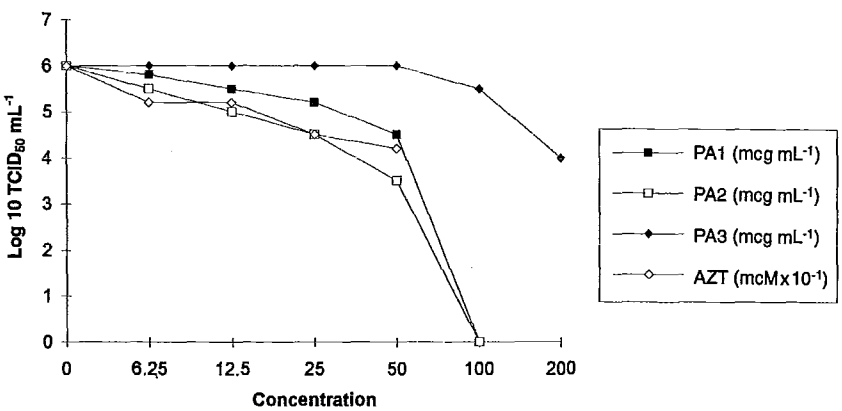

Fig. 2. Inhibitory effect of different concentrations of extracts of *Persea americana* on HIV-1 replication.
H9 cells were treated with different concentrations of extracts or no extract (virus control). The drug treated H9 cells were infected with 10-fold dilutions (10^{-1}–10^{-7}) of HIV-1 RF in six replicates using a 96-well plate. The number of giant cells was recorded from day 1 to day 8 by microscopic examination and the $TCID_{50}$ of the virus in the test and control experiment was calculated. The experiment was repeated three times with similar results. PA2 = methanol insoluble extract.

Table 1. Effect of different infectivity doses ($TCID_{50}$) of HIV-1 RF on the ED_{50} of extracts and fractions of *P. americana*

Extract[a]	ED_{50} ($\mu g\ mL^{-1}$) preventing infection by 10 $TCID_{50}$	100 $TCID_{50}$
PA1	18.7	33.7
PA2	9.3	12.5
PA3	100.0	150.0
PA2a	48.7	70.5
PA2b	6.2	10.3
PA2c	8.4	9.3
PA2d	–[b]	–[b]

[a]PA1 = crude extract; PA2 = methanol insoluble extract; PA3 = methanol soluble extract; PA2a, b, c, d = different fractions obtained from PA2.
[b] No activity.

flavonoids which have direct antiviral effects (Hudson 1990). *P. americana* leaves contain, in addition to the flavonoid quercitrin, other flavonoids such as apigenin, luteolin and astragalin (Santos *et al.*, 1988; Mericli *et al.*, 1992) that could be responsible for the virucidal effect of the PA1, PA2, PA2b and PA2c extracts. On the other hand, other compounds such as perseitol (D-glicero-D-galacto-heptitol) (Santos *et al.* 1988), essential oils and gama-carotene (Montes *et al.*, 1981) were also isolated from *P. americana* leaves and could be involved in the process of virus inhibition. Polyphenolic compounds, such as tannic acid, are frequently found in extracts of higher plants and have broad antiviral spectra, inactivating a variety of virions extracellularly (Cheng and Yeung, 1988). Additionally, Animashaun *et al.* (1993) have shown that lectins from plants have the ability to prevent HIV-1 infection while Mead *et al.* (1980) have described a lectin-like activity from *P. americana* seeds.

Previous results (Wigg, 1991) have shown that the aqueous crude extract of *P. americana* and one fraction obtained from the methanol soluble extract was active *in vitro* against adenovirus (a member of the Rhabdoviridae) type 3 but was without any antiviral effect vs. vesicular stomatitis virus (Rhabdoviridae) and western equine encephalomyelitis (Togaviridae). The extract showed virucidal activity and inhibited the initial steps of the replicative cycle of the virus without any interference with the viral DNA synthesis. Recently, Miranda (1995) has shown the inhibitory effect of this extract on different strains of aciclovir-resistant herpes simplex virus type 1 (HSV-1). Koseki *et al.* (1990) have also demonstrated the effect of the crude extract of *P. americana* against Aujeszky disease virus (ADV) (Herpesviridae) and bovine virus diarrhoea (BVD) (Flaviviridae).

It has been demonstrated for the first time that extracts of *P. americana* species have moderate anti-HIV activity, including moderate virucidal effects, *in vitro*. The molecular composition of the extracts is likely to be complex. We are particularly interested in identifying active constituents with virucidal activity. At present the only compound in clinical use as an anti-HIV virucide is the spermicide surfactant compound nonoxynol-9 (Malkovsky *et al.*, 1988; Polsky *et al.*, 1988). Further virucidal molecules are urgently required since the latter compound exerts simultaneous toxic effects on the vaginal mucosa, possibly resulting in enhanced infection by HIV-1 in women self-administrating the compound (Kreis *et al.*, 1992).

Materials and Experimental Procedures

Plant extract

Leaves of *Persea americana* (PA) collected in Bahia, Brazil were identified and deposited at the Nucleo de Pesquisa de Produtos Naturais, UFRJ, Brazil. Powdered dried leaves were extracted by infusion in boiling distilled water (10% w/v) and subsequent cooling to about 60 °C. The extract was then filtered through paper. Aliquots of this extract (PA1) were immediately separated, frozen, further lyophilized and stored at –20 °C.

Fractionation of the extract

The lyophilized extract (PA1) was exhaustively washed with aliquots of methanol at room temperature using magnetic stirring. The methanolic phases were combined together and evaporated until dry (PA3). The insoluble residue (PA2), made by drying in a desiccator until constant weight, was solubilized in water (10% w/v). Two fractions were obtained, only one of which was water soluble. This water soluble fraction (90% of PA2) was frozen, lyophilized and chromatographed on a reverse-phase column (29 x 4 cm) using a silanized silica 60 gel as support (70–230 mesh, Merck). Eluting with an increasing concentration gradient of methanol in water gave two

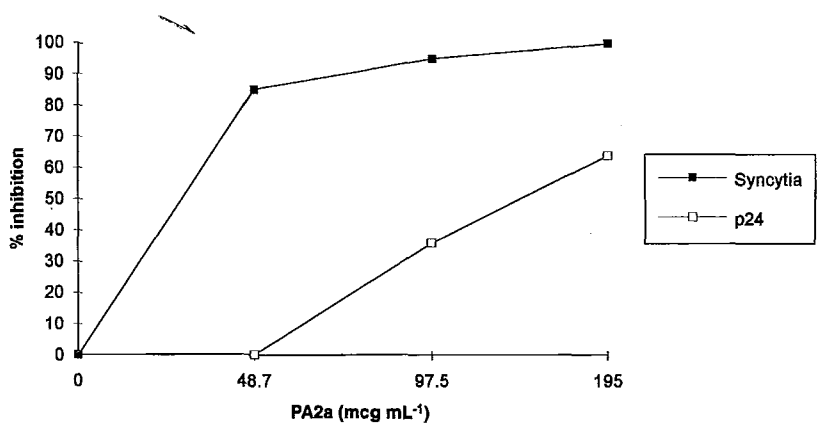

Fig. 3. Inhibitory effect of the PA1 and PA2a fractions of *P. americana* on HIV-1 RF replication shown by release of viral p24 antigen and virus induced syncytia.
H9 cells were treated with five concentrations of the PA1 or PA2a fraction or control medium for 60 min at 37 °C as described in Materials and Experimental Procedures. At day 8 the number of virus induced syncytia was counted and cell supernatants collected for quantification of viral p24 antigen.

Table 2. Virucidal activity of the extracts and fractions of *P. americana*

Extract[a] ($\mu g\ mL^{-1}$)		log_{10} $TCID_{50}$ reduction in virus titre
PA1	(200)	4.5
PA2	(200)	4.5
PA2a	(195)	0
PA2b	(200)	4.5
PA2c	(200)	4.5
PA2d	(200)	0

[a]PA1 = crude extract; PA2 = methanol insoluble extract; PA2a, b, c, d = different fractions obtained from PA2.
Compounds were incubated with virus for 1 h at 37°C and tested for residual infective virus. Virus control titre 10^6 $TCID_{50}$ mL^{-1}.

fractions (PA2a, b). After elution with 100% methanol, the column was washed with a methanolic TFA 0.5% solution. The insoluble fraction (10% of PA2) was further washed with methanol with constant agitation. The soluble part was concentrated until dry, resuspended in methanol and filtered through paper. After dissolving in distilled water, the residue (PA2c) and the filtrate (PA2d) were evaporated under vacum until dry. All chromatography processes were monitored on TLC (precoated silica gel 60 F_{254} plates, Merck, UK) using BAW 8:1:1 as solvent system. The chromatograms were visualized under UV (short and long waves). The enriched-flavonoid fraction (PA2a) was partially purified on a Sephadex LH-20 (Pharmacia St. Elpans, UK) chromatography column (19.5 x 2.5 cm) in a reverse-phase system (gradient water and methanol) to give quercitrin (quercetin-3-O-α-L-rhamnopyranoside) and another compound not yet identified. The structure of quercitrin compound was deduced from the analysis of ^1H (Cosy $^1H - ^1H$) and 13C NMR spectra in a Brucker AC 300 spectrometer in CD_3OD-D_2O.

Preparation of the extracts and fractions

The extracts and fractions were dissolved in water or DMSO (0.1%), immediately diluted in RPMI-1640 medium and filtered through a 0.22 μM Millipore membrane.

Cells and viruses

H9 cells were maintained in RPMI-1640 medium supplemented with growth medium (10% fetal calf serum, 50 units mL^{-1} penicillin, 50 $\mu g\ mL^{-1}$ streptomycin, 2 mM L-glutamine and 25 mM HEPES buffer) at 37 °C in a 5% CO_2 atmosphere. Virus stock (HIV-1 RF strain) was prepared from 7-day harvests of cell-free culture supernatant of infected H9 cells and aliquots were stored in liquid nitrogen. The virus stock had an infectivity titre of 10^6 $TCID_{50}$ mL^{-1} as determined by terminal dilution.

Cytotoxicity evaluation

180 μL of 4 x10^5 H9 cells mL^{-1} were incubated with 20 μL of medium or two-fold serial dilutions of extracts at 37 °C. Each extract was tested in duplicate using 96-well plates. At days 5 and 8 after the adding of the extracts, the number of viable cells was counted and the percentage in each well was calculated using the trypan blue-exclusion method. The mean number of viable cells for each concentration and the time point was calculated, and the result plotted as the percentage of viable cells vs. the concentration of the extracts. The highest concentration of an extract which showed no cytotoxicity was taken as the non-toxic concentration and used in the antiviral tests.

Antiviral assay

The antiviral effect of the extracts was determined by the inhibition of virus induced syncytia. H9 cells at concentration of 4 x10^5 viable cells mL^{-1} were incubated with different concentrations of extracts or no extract (virus control) for 1 h at 37 °C. The treated H9 cells were infected with 10-fold dilutions of HIV-1 RF (10^6 $TCID_{50}$ mL^{-1}) in six replicates using 96-well plates and the plates incubated at 37 °C. The presence of giant cells (syncytia) was recorded on a daily basis by microscopic examination. At day 8, the virus titre was calculated by the 50% tissue culture-infective dose ($TCID_{50}$) method. In parallel to the extract to be tested, AZT (range 0.6–5 μM) was used as a positive control. The relatively high concentration of AZT is required to inhibit viral replication in this particular assay system using H9 cells. To estimate the reduction of the infectivity of HIV-1 RF, after treatment with PA1 and PA2 extracts, the supernatants of the antiviral test were harvested and titrated for infective virus in H9 cells (Lagrota *et al.*, 1994). In another experiment, 10 and 100 $TCID_{50}$ of HIV-1 RF were used to infect the drug treated or untreated cells and the 50% effective-dose (ED_{50}), namely the concentration of extract or fraction required to reduce virus yield by 50%, was determined by extrapolation.

Virustatic assay

In the specific case of the PA2a fraction, H9 cells at a concentration of 2 x 10^6 viable cells mL^{-1} (450 μL) were incubated with three non-toxic concentrations (195; 97.5 and 48.7 $\mu g\ mL^{-1}$) of this fraction (50 μL) or medium for 60 min at 37 °C. The treated H9 cells were washed and then infected with 10 $TCID_{50}$ mL^{-1} of HIV-1 RF. After 3 h of incubation at 37 °C, 15 mL of RPMI medium was added to each tube containing viruses and fraction dilutions. The cells were washed twice to remove excess p24 antigen. Cell pellets were resuspended using 2 mL of growth medium, split in two wells of a 48-well plate with addition of the appropriate concentration of the PA2a fraction. The presence of virus induced syncytia was recorded daily up to day 8 by microscopic examination, and the percentage inhibition of syncytia formation was calculated. At days 5 and 8, 200 μL of the cell supernatants were collected for quantification of viral p24 antigen.

Viral p24 antigen assay

Cell supernatants were first treated with 0.1% Empigen and then tested by ELISA using a commercial kit (Coulter Electronics Ltd, UK) for the presence of the viral p24 antigen. Viral antigen concentration in excess of 10 ng mL^{-1} were considered as 100% viral production.

Virucidal assay

HIV-1 RF ($\approx 10^6$ $TCID_{50}$ mL^{-1}) was incubated for 1 h at 37 °C with the appropriate extract, fraction or medium as a control. All drug samples were used at a concentration of 200 μg mL^{-1} except the PA2a fraction which contained 195 μg mL^{-1}. After incubation, the treated virus preparations were diluted (10-fold dilutions) and inoculated into individual wells of a 96-well plate containing H9 cells at a concentration of 4×10^5 viable cells mL^{-1}. Cultures were observed daily for syncytium-formation and the residual virus infectivity was expressed as a $\log_{10}$ reduction in virus titre (Lagrota *et al.*, 1994).

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